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FOREWORD

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INTRODUCTION

Background

Degradation of the extracellular matrix (ECM) by proteases is a fundamental characteristic of normal tissue remodeling in physiological processes such as angiogenesis, wound healing and macrophage migration etc. However, it is well established that the pathological process of tumor cell metastases also involves the presence of several different classes of secreted and membrane bound proteases at the cell-matrix interface (1-9). It has also been shown that tumor cells elaborate extracellular ventral membrane protrusions termed "invadopodia" at the cell-matrix interface upon which certain membrane and secreted proteases are reported to be localized (10-12). Indeed, there appears to be a direct correlation between invasiveness and the elaboration of invadopodia which dissolve the surrounding matrix.(13). Furthermore, by utilizing an *in vitro* assay system that consists of growing cells on fluorescence labeled or radiolabeled ECM components that are covalently crosslinked to gelatin film, we can detect the elaboration of invadopodia by human cancer cell lines (14).

Using this *in vitro* extracellular matrix (ECM) degradation/invasion assay our laboratory identified a highly invasive melanoma cell line, LOX, that had previously been shown to have a high incidence of lung metastasis in athymic mice (15). LOX cells express a 170 kDa membrane-bound gelatinase, seprase, whose expression correlates with LOX invasiveness in the *in vitro* ECM degradation/invasion assay. Seprase expression was also detected in the breast carcinoma cell line MDA-MB 436 and more recently in the breast carcinoma line Hs 578T and the normal myoepithelial line Hs 578Bst. It is a homodimer consisting of 97 kDa subunits that are proteolytically inactive (17). Sequence analysis of 3 internal proteolytic fragments of the 97-kDa polypeptide revealed highest homology (67-88%) to the 95-kDa fibroblast activation protein α (FAP α), an inducible cell surface glycoprotein which is reported to be expressed on reactive stromal fibroblasts of epithelial cancers including breast cancer and healing wounds but whose function is unknown(18), and to a lower extent (33-70%) with the 110-kDa subunit of dipeptidyl peptidase IV (DPPIV). Dipeptidyl peptidase IV is a multifunctional protein that has wide tissue distribution and is a member of the nonclassical serine hydrolase family (19-21). Like the 170-kDa seprase its protease activity is dependent on the association of its 110 kDa subunits (22,23). We reported last year (24) the molecular cloning of a cDNA from LOX that encodes the 97-kDa subunit of seprase. Its deduced amino acid sequence confirmed the above mentioned peptide sequence data and revealed that seprase is a type II integral membrane protein with a cytoplasmic tail of 6 amino acids, followed by a transmembrane domain of 20 amino

acids and an extracellular domain of 734 amino acids. And that its carboxy terminus contains a catalytic region (~200 amino acids) which is homologous (68% identity) to that of DPPIV. We also sequenced seprase cDNA isolated from the breast carcinoma cell line MDA-MB-436 which revealed no significant difference in their primary structures. However, the seprase cDNA clones did show what appeared to be a significant sequence divergence (45 contiguous amino acids) in their catalytic regions when compared to deduced amino acid sequence predicted by the FAP α cDNA (18; see Body of Report). Otherwise, seprase and FAP α were found to be highly homologous (94% identity). We also showed that the seprase cDNA encoded a functional 170-kDa gelatinase in transiently transfected COS-7 cells.

Purpose of present work

We initially proposed to determine the possible role of seprase in the metastasis of breast cancer. The *in vitro* breast carcinoma line MDA-MB-436 which is known to express seprase would appear to be a good candidate for transfection experiments with sense and antisense cDNA constructs that encode 97-kDa subunit of seprase. Using the *in vitro* ECM invasion/degradation assay (Introduction) and also a newly developed *in vitro* 3 dimensional collagen degradation assay (see Body) we could monitor the effects of the overexpression and underexpression of this gelatinase on the invasive phenotype of MDA-MB-436 as well as its effects on other seprase negative cell lines (MCF-7, MDA-MB-231 etc.) at various stages of neoplastic development. Thus the results from these experiments might suggest and/or confirm a role for this membrane bound protease in the invasion of the ECM.

Scope of research

Our initial proposal to determine the possible role of seprase in the metastasis of breast cancer was to involve the isolation and sequence determination of seprase cDNAs, transfection studies on breast carcinoma cell lines and if possible mutagenesis studies on seprase subunit association. Importantly and unexpectedly, in the course of carrying out this grant proposal we have identified an alternatively spliced seprase mRNA transcript which potentially encodes an intracellular protease (see Body) which would therefore be distinct in function and size from the 170-kDa membrane bound form.

BODY

Experimental methods

Reverse Transcriptase - Polymerase Chain Reaction - Total RNA was isolated from tumor cells using the RNA Stat-60 kit (Tel-Test "B", Inc.). Six µg of total RNA were used as template for first strand cDNA synthesis with oligo (dT)₁₂₋₁₈ as primer. The reaction was catalyzed by Superscript II Rnase H⁻ reverse transcriptase as directed by the manufacturer (Gibco/BRL). Ten oligonucleotide primers were synthesized corresponding to sense and antisense orientations based on the published FAPα cDNA sequence (18). The primers were utilized for both polymerase chain reaction (PCR) and DNA sequence analysis (see below). The 6 sense primers and their corresponding FAPα nucleotide positions were: FAP-1 5'-CCACGCTCTGAAGACAGAATT-3' (#161-181; 5' untranslated region (UTR)); FAP-3 5'-CCAGCAATGATAGCCTCAA-3' (#1055-1073); FAP-5 5'-TGACAAACTCCTCTATGCAG-3' (#1951-1971); FAP-8 5'-TCCAAGCAAGAAGTGTGTTA-3' (#1507-1526); FAP-11 5'-TTACATCTATGACCTTAGCA-3' (#598-617); FAP-13 5'-GAAACTTGGCACGGTATTCAA-3' (#45-65; 5' UTR). The 4 antisense primers and their corresponding nucleotide positions were: FAP-4 5'-ACAGACCTTACACTCTGAC-3' (#1863-1845); FAP-6 5'-TCAGATTCTGATACAGGCT-3' (#2523-2505; 3' UTR); FAP-10 5'-TAACACACTTCTTGCTTGGA-3' (#1526-1507); FAP-12 5'-AACACTGTGTCCAAAGCAA-3' (#2731-2713; 3' UTR). In addition, an oligonucleotide primer corresponding to the T7 promoter of the pCR3.1 vector (Invitrogen) was synthesized: 5'-TAATACGACTCACTATAGGG-3'. Polymerase chain reactions were carried out using 2 µl of the reverse transcriptase reactions and recombinant TAQ polymerase using the manufacturers' protocol (Gibco/BRL) on a Perkin Elmer Gene Amp 9600 Cycler. One PCR cycle: Denaturation at 94 °C for 30 s; Anneal at 55 °C for 20 s and Extend at 72 °C for 30 s for a total of 40 cycles.

DNA Cloning - A ~1.2 kb amplicon generated with the FAP 1+6 primers was isolated from a 1% agarose gel using the Qiagen gel extraction kit. Purified cDNA (20ng) was ligated to 30 ng of the pCR3.1 mammalian expression vector. Ligation, transformation and selection of recombinant clones were carried out using the Eukaryotic TA Cloning Kit from Invitrogen. Purified recombinant plasmid DNA was obtained using Qiagen Tip 20 and Tip 500 columns.

DNA Sequence Analysis - Cycle sequencing reactions were carried out using the ABI PRISM Dye Terminator Ready Reaction Kit (Perkin Elmer). Automated sequencing was carried out on an ABI PRISM 377 DNA sequencer.

In Vitro Transcription - *In vitro* transcription of seprase cDNA was carried out using the T7 Cap-Scribe Kit following the manufacturers' instructions (Boehringer Mannheim). Both linearized plasmid and PCR generated amplicons using the T7 and FAP-6 or FAP-12 primers were used as templates for RNA transcription.

In Vitro Translation - *In vitro* translation was carried out using both Wheat Germ Extract and the Flexi Rabbit Reticulocyte Lysate System (Promega) following the

manufacturers' protocol. The final potassium and magnesium ion concentrations were set to 73mM and 2.1mM for the wheat germ extract translation and 86mM and 1.2mM for the rabbit reticulocyte translation. Translation products were labeled with L-[4,5-³H] leucine (152Ci/mmol) from Amersham.

Coupled Transcription-Translation – Coupled *in vitro* transcription and translation was carried out using the TnT T7 coupled reticulocyte lysate system from Promega as described. Plasmids pA11 (vector), pA15 (full length seprase; FAP 1+6), pA12 (truncated seprase; FAP 1+6) and p1425 (truncated seprase; FAP 12+13) were used at a concentration of ~1µg per 25µl reaction.

Stable Transfection of In Vitro Breast Carcinoma Cell Lines – Stable transfection of the breast carcinoma lines MCF-7, MDA-MB-436 and MDA-MB-231 were carried out with the plasmids (see above) pA11, pA15 and pA12 using the lipofectamine reagent from Gibco/BRL following the manufacturers protocol except for the following modifications:

- A) The complete growth medium was "T" medium – 1:1 RPMI + DMEM (Gibco/BRL), 10% Bovine calf serum (HyClone), 5% Nu-Serum IV (Collaborative Biomedical), 1% L-glutamine (Gibco/BRL) and 1% Penicillin and streptomycin sulfate (Gibco/BRL).
 - B) The stable transfection selection medium consisted of "T" medium + G418 at concentrations of 500 µg/ml (MCF-7), 300 µg/ml (MDA-MB-436) and 800 µg/ml (MDA-MB-231).
 - C) Solution A – 1.5 µg of each plasmid; Solution B – 5µl of Lipofectamine reagent.
 - D) Solution A+B was incubated 30 minutes.
 - E) The DNA-liposome complex was incubated with the cells for 6 hours.
- All other procedures for isolating stable transfectant clones were as described (25).

Procedures, Results and Discussion

Seprase and FAP α are the same protein

In our midterm report (see Introduction) we stated that seprase and FAP α exhibited a significant difference in their catalytic regions that was due to the insertion of 3 guanine nucleotides in the region #1876-G to #2010-G in seprase and that FAP α because of this sequence divergence might not be a functional protease. The possibility that this divergence in sequence was due to alternative RNA processing was considered unlikely when we examined the genomic organization of the human (26) and mouse (27) DPPIV genes. We found that the corresponding genomic sequences in the DPPIV genes that would encode the region of divergence (Gly⁶²⁶ to Lys⁶⁷⁰) are contained within 2 contiguous exons (exon 22 and 23) but that the divergence in sequence does not align with the exon-intron junctions. Furthermore, the high degree sequence identity (80%) and the lack of any insertions or deletions of amino acids in both the seprase and DPPIV cDNA sequences that are encoded by exon 22 and 23 suggests that no exon-intron junction sliding has occurred between the seprase and DPPIV genes. Additionally, we sequenced the region of divergence in 13 other cDNA clones including one clone from the WI-38 fibroblast cell line. All clones exhibited the insertion of 3 guanine nucleotides that correspond with the seprase cDNA sequence. That seprase and FAP α are identical in this region was confirmed this year when the FAP α cDNA sequence was updated

(GenBank U09278). Although 3 nonconservative amino acid substitutions at positions #207 (Pro to Ala), #229 (Lys to Thr) and #354 (Arg to Thr) remain to be resolved it is clear that seprase and FAP α are the same protein.

Seprase RNA undergoes alternative splicing

In the process of cloning a cDNA by RT-PCR of LOX RNA that would encode the complete open reading frame (ORF) of seprase we detected 2 major bands (at ~ 2.4 and 1.2 kb) when using the primer pair FAP 1+6 which corresponds to sequences within the 5' UTR (FAP 1) and 3' UTR (FAP 6) of seprase mRNA (Fig. 1, lane 6, pg.18). Indeed, upon further investigation by scanning the length of the seprase mRNA by RT-PCR with primers that correspond to sequences that begin at nucleotide positions # 45 (FAP 13), # 161 (FAP 1), # 598 (FAP 11), # 1055 (FAP-3), # 1526 (FAP 10), #1863 (FAP-4) and # 2523 (FAP-6) we found that primers FAP-11 and FAP-3 only generated single bands (Fig. 1, lanes 3,5 and 7) while the other primers generated two major bands (Fig.1, lanes 1,2,4 and 6). These results suggested that a nucleotide region between #161 and # 1526 that is ~ 1.2 kb in length is deleted in some of the seprase RNA transcripts.

This finding was confirmed when we sequenced several independent clones from LOX and 2 from the breast carcinoma line MDA-MB-436. In fact, this truncated transcript has been detected (by RT-PCR) in every cell line, normal or pathological, that expresses seprase including Hs 578Bst and its malignant counterpart Hs 578T (see below). The nucleotide sequence of the truncated RNA transcript is presented in Fig. 2 (pg.19). The sequence indicates that alternative exon splicing is occurring in the seprase pre-mRNA transcript which obeys the consensus sequence motifs for splice donor and receptor sites (28). The nucleotide sequence corresponding to positions #221 to #1443 (# 13 to #1235 for the ORF; Fig. 2) is deleted which causes a frame shift in the ORF leading to the premature termination of full length seprase as a pentapeptide. Interestingly, the region of the seprase mRNA which is deleted in this truncated transcript would normally encode the cytoplasmic tail, transmembrane region, membrane proximal N-glycosylation and Cys rich regions. However, the sequence encoding the catalytic domain (# 1678 to # 2280 for the ORF) remains intact.

Analysis of the truncated mRNA downstream of the premature termination codon for full length seprase indicated that the first AUG codon that had a favorable sequence for initiation of translation (29) and that was not followed by a premature termination codon was in the seprase reading frame at positions # 1564 to # 1566 for the full length ORF. Importantly, if this downstream AUG codon was functional, the structure of the truncated mRNA would resemble a subset of mRNAs that are known to encode oncogenes, transcription factors, signal transduction components etc. that possess an upstream minicistron which is followed by a downstream coding sequence (30,31). To test the hypothesis that the truncated mRNA reinitiated protein synthesis at this downstream AUG codon we carried out *in vitro* translation with the truncated RNA (see Materials and Methods) using wheat germ extract and rabbit reticulocyte lysate. As can be seen in Fig. 3, lanes 3,4,6,7 and 8 (pg.20) both the wheat germ (lanes 3 and 4) and Flexi rabbit reticulocyte lysate (lanes 6 and 7) systems exhibit 2 bands at ~ 26 and 28 kDa. The coupled transcription –translation assay with plasmids pA12 and p1425 that encode the truncated mRNA produce a very strong band at ~ 28 kDa. The size of the *in vitro* translation products generated in this experiment are in good agreement with the predicted size of ~27 kDa for the downstream translation product. Additionally,

computer analysis of all possible ORFs in the truncated cDNA indicated that the next largest ORF would also be in the seprase reading frame and would encode a protein of ~22 kDa.

Using RT-PCR we have examined several *in vitro* cell lines qualitatively for the production and relative abundance of the 2 seprase mRNA transcripts. As mentioned above, the induction of the truncated transcript is not specific for either normal or malignant cell lines but is produced in all cell lines that we have examined that express seprase. However, in examining the Hs 578Bst (normal, myoepithelial; Fig. 4 (pg.21), lanes 1 and 2) and the Hs 578T (carcinoma; Fig. 4, lanes 3 and 4) *in vitro* cell lines, grown in the presence or absence of medium containing serum, we obtained a strong signal differential for the truncated transcript. It should be noted that we have carried out RT-PCR with other RNA preparations from these cell lines with less signal differential. Nevertheless, it appears that in Hs 578T there is a trend toward increased production of the truncated transcript in the presence of medium containing serum (Fig. 4, lane 3).

Stable transfection of breast carcinoma cell lines

We have isolated stable transfectants (see Materials and Methods) for the plasmids pA11 (vector), pA15 (full length seprase) and pA12 (truncated seprase) for 3 different breast carcinoma lines: MCF-7 (seprase negative, low *in vitro* invasiveness (32)), MDA-MB-436 (seprase positive, moderate *in vitro* invasiveness) and MDA-MB-231 (seprase negative, high *in vitro* invasiveness). Additionally, we have also isolated a stable transfectant for the MDA-MB-436 cell line that was transfected with pA26 (seprase cDNA in antisense orientation). We examined the transfected cell lines using RT-PCR and Western blot analyses to confirm the expression of seprase.

Using the oligonucleotide primers FAP 1+4, we detected the presence of the full length transcript in MCF-7 cells (Fig. 5, lane 3; pg.22) transfected with pA15 but it was absent in the mock (pA11) transfected MCF-7 cells (Fig. 5, lane 2). Likewise, we found the same result in MDA-MB-231 cells that had been transfected with pA15 (Fig. 5, lane 5) and pA11 (Fig. 5, lane 4). Also, the MCF-7 cells transfected with pA15 (Fig. 5, lane 3) appear to show the presence of the truncated transcript. Thus it would appear that the full length transcript generated from the pA15 cDNA that is integrated into the MCF-7 genome undergoes alternative exon splicing. RT-PCR analysis of MDA-MB-436 cells that were transfected with pA11 (Fig. 5, lane 6), pA26 (lane 7) and pA15 (lane 8) is less clear cut. This cell line exhibits the endogenous expression of seprase and thus shows 2 major bands with the FAP 1+4 primer pair in the mock transfected cells (lane 6). The pA15 transfected cell line appears to show a slight increase in the full length transcript but clearly not a marked increase in this transcript. The pA26 transfected cells (lane 7) unexpectedly appear to show a decrease in both the endogenous full length and truncated mRNAs.

We also examined the stable transfectants for the expression of the 170-kDa seprase by Western blot analysis (Fig. 6, pg.23). Seprase expression correlated with the RT-PCR results obtained for the MCF-7 pA15 transfectant (lane 5) and the MCF-7 mock transfected cells (lane 4). Likewise, MDA-MB-231 transfected with pA15 (lane 3) expresses seprase but the mock transfected cells do not (lane 2). As was the case with RT-PCR, the expression results with MDA-MB-436 is less clear cut. The mock transfected cells (lane 6) as expected express endogenous seprase; however, the pA15 transfected cells (lane 7) in agreement with the RT-PCR results exhibit either the same or

a slightly greater level of seprase but certainly not a marked increase in seprase expression. Finally, the pA26 transfected cells (lane 8) do not show any indication of decreased seprase expression. The reason for the failure of this stable transfectant to suppress endogenous seprase mRNA translation is unknown.

We now plan to test these stable transfectants in *in vitro* matrix degradation/invasion assays. Our laboratory is currently developing a new *in vitro* assay system in which the transfected cells will be encapsulated in a 3-D collagen network which more closely resembles the *in vivo* matrix environment. It will allow us to measure any change in the invasive phenotype of cells transfected with the pA15 plasmid that encodes 170-kDa membrane form of seprase. We are beginning to test the various stable transfected lines simultaneously with the submission of this report.

CONCLUSION

LOX cells express a 170 kDa membrane bound gelatinase, seprase, whose expression correlates with LOX invasiveness in the *in vitro* ECM degradation/invasion assay (16). We initially proposed to identify the 170 kDa membrane bound protease, seprase, by isolating a cDNA clone that encoded its 97 kDa subunit. And we were also interested in determining its potential role in breast cancer metastasis. As a result of our research over the period of this grant we can now definitively describe this protease at primary structure level. Its deduced amino acid sequence clearly identifies it as a member of the nonclassical serine hydrolase family. Furthermore, its high degree of identity (52%) with the exopeptidase DPPIV and the fact that both proteases require subunit association for biological activity suggests that they represent a new subfamily of serine integral membrane proteases (SIMP). Importantly, determination of the deduced amino acid sequence of seprase has clarified and confirmed its relationship to FAP α which is reported to be expressed on reactive stromal fibroblasts of various carcinomas including breast cancer but whose function was formerly unknown. We now know that seprase and FAP α are in fact the same protein. Also, the tissue expression pattern of FAP α may be different from what was formerly thought. It had not been found to be expressed on *in vitro* carcinoma cell lines (33), however, we have identified two carcinoma cell lines, MDA-MB-436 and Hs 578T, that both express seprase/FAP α . It therefore remains to be determined if the *in vivo* expression pattern of seprase/FAP α is limited to stromal fibroblasts in carcinomas. We have now produced stable seprase transfectants of breast cancer cell lines that represent different levels of neoplastic development in terms of *in vitro* invasiveness and we will now attempt to determine if expression of membrane bound seprase/FAP α changes their metastatic/invasive potential. Unexpectedly, we have made what appears to be a significant finding concerning the molecular biology of seprase/FAP α . The seprase pre-mRNA undergoes alternative exon splicing giving rise to a truncated mRNA that cannot encode the membrane form of seprase/FAP α . The existence of this truncated mRNA would appear to represent a cellular down regulation mechanism for seprase which is carried out at the level of RNA splicing. Although the induction of seprase RNA alternative processing is not tumor cell specific, preliminary results from the cell lines Hs 578Bst and Hs 578T suggest that their may be an up regulation of the truncated mRNA in the tumor cell line. Importantly, sequence analysis of the truncated mRNA indicates that a potential ORF exists downstream of the prematurely terminated seprase full length ORF. This downstream ORF would encode a truncated intracellular form of seprase (MW ~27 kDa) that would include the entire catalytic region of the full length membrane form. *In vitro* translation of the truncated mRNA confirms that the downstream ORF exists. We will now attempt to isolate the intracellular form of seprase and elucidate its role in the biology of seprase/FAP α .

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Abstracts

1. L.A. Goldstein, G. Gherzi, M.L. Pineiro-Sanchez, M. Salamone, Y. Yeh, D. Flessate, W.-T. Chen, *Molecular Cloning of Seprase: A Serine Integral Membrane Protease from Human Melanoma*, DOD Breast Cancer Research Program: An Era of Hope, Renaissance Hotel, Washington, D.C. (1997).

Salaried Personnel

1. Leslie A. Goldstein

APPENDIX

Figure Legends

Fig. 1. RT-PCR analysis of LOX RNA using FAP α primers. RT-PCR was carried out on LOX total RNA using FAP α primers that correspond to sequences within the 5' UTR, ORF and 3'UTR of seprase/FAP α cDNA (see Materials and Methods). Lane 1 – FAP 13+4; lane 2 – FAP1+4; lane 3 – FAP11+4; lane 4 – FAP 13+10; lane 5 – FAP 11+10; lane 6 – FAP 1+6; lane 7 – FAP 3+6; lane 8 – size markers.

Fig. 2. Nucleotide and deduced amino acid sequence of seprase mRNA splice variant. Nucleotide sequence of full length seprase which encodes complete ORF is shown. Underlined sequence represents region which is deleted in the truncated splice variant. Bold nucleotides (#1561, 1564-1567) represent downstream translation initiation site (29). The putative catalytic triad of truncated seprase (Ser¹⁰³, Asp¹⁸¹, His²¹³) and its protease consensus motif are represented by bold underlined amino acids.

Fig. 3. *In vitro* translation of truncated seprase splice variant. *In vitro* translation was carried out using full length or truncated seprase RNA transcripts generated by *in vitro* transcription and plasmids encoding truncated seprase RNA (coupled transcription-translation) in wheat germ and rabbit reticulocyte systems. See Materials and Methods for details. Lane 1 – full length transcript in wheat germ system; lane 2. – minus RNA wheat germ control; lane 3 – truncated *in vitro* transcript generated from plasmid p1425; lane 4 – same as lane 3 but plasmid pA12; lane 5 – minus RNA rabbit reticulocyte control; lane 6 – truncated *in vitro* transcript generated from p1425 in rabbit reticulocyte system; lane 7 - *in vitro* translation product generated from p1425 using coupled transcription-translation rabbit reticulocyte system; lane 8 – same as lane 7 but plasmid pA12.

Fig. 4. RT-PCR analysis of Hs 578Bst and Hs 578T RNA for seprase transcripts. RT-PCR analysis was carried out on Hs 578Bst and Hs 578T total RNA using the primer pair FAP 1+4 which is capable of detecting both the full length and truncated seprase mRNAs. Note that the minor band at ~1 kb in lanes 1-4 is an artifact of PCR. Lane 1 – Hs 578Bst RNA from cells cultured in medium plus serum; lane 2 – same as lane 1 but minus serum; lane 3 – Hs 578T RNA from cells cultured in medium plus serum; lane 4 – same as lane 3 but minus serum; lane 5 – minus reverse transcriptase negative control; lane 6 – size markers.

Fig. 5. RT-PCR analysis of RNA from MCF-7, MDA-MB-231 and MDA-MB-436 stable transfectants. RT-PCR analysis was carried out on total RNA isolated from stable transfectants generated with plasmids pA11 (vector), pA15 (full length seprase) and pA26 (antisense full length seprase) using the primer pair FAP 1+4. Lane 1 – LOX RNA positive control; lane 2 – MCF-7 transfected with pA11; lane 3 – MCF-7 transfected with pA15; lane 4 – MDA-MB-231 transfected with pA11; lane 5 – MDA-

MB-231 transfected with pA15; lane 6 – MDA-MB-436 transfected with pA11; lane 7 – MDA-MB-436 transfected with pA26; lane 8 – MDA-MB- 436 transfected with pA15.

Fig. 6. Western blot analysis of MCF-7, MDA-MB-231 and MDA-MB-436 stable transfectants. Western blot analysis was carried out on the detergent lysates of stable transfectants produced with the plasmids pA11 (vector), pA15 (full length seprase) and pA26 (antisense full length seprase) using the monoclonal antibody D8 (16) which is specific for the 170 kDa dimeric form of seprase. Lane 1 – wheat germ agglutinin purified LOX extract (positive control); lane 2 – MDA-MB-231 transfected with pA11; lane 3 – MDA-MB-231 transfected with pA15; lane 4 – MCF-7 transfected with pA11; lane 5 – MCF-7 transfected with pA15; lane 6 – MDA-MB-436 transfected with pA11; lane 7 – MDA-MB-436 transfected with pA15; lane 8 – MDA-MB-436 transfected with pA26.

Figure 1

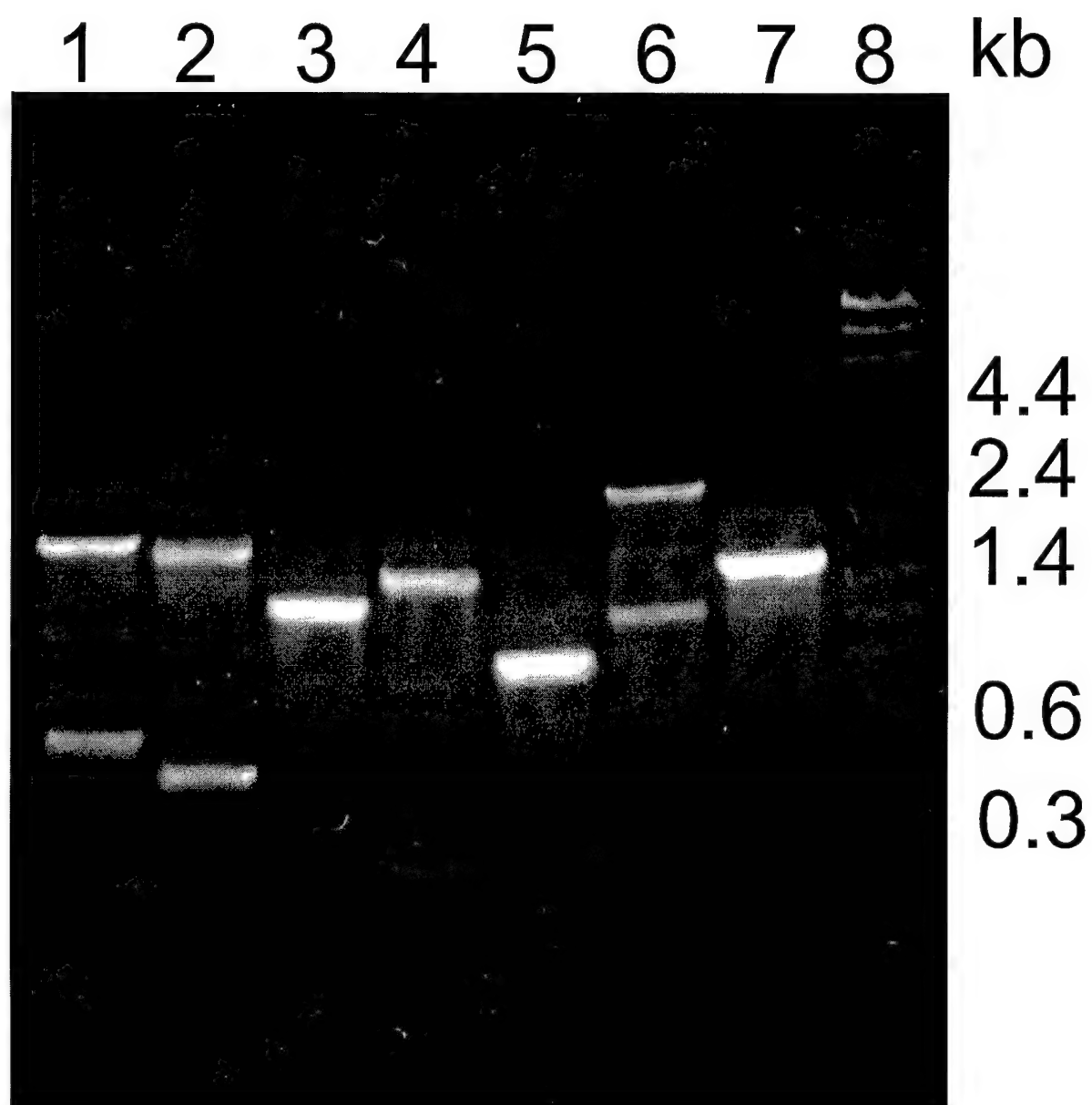


Figure 2

ATG	AAG	ACT	TGG	GTA	AAA	ATC	GTA	TTT	GGA	GTT	GCC	ACC	TCT	GCT	GTG	CTT	GCC	TTA	TTG	GTG	ATG	TGC	ATT	GTC	75		
M	K	T	W																						4		
TTA	CGC	CCT	TCA	AGA	GTT	CAT	AAC	TCT	GAA	GAA	AAT	ACA	ATG	AGA	GCA	CTC	ACA	CTG	AAG	GAT	ATT	TTA	AAT	GGA	150		
ACA	TTT	TCT	TAT	AAA	ACA	TTT	TTT	CCA	AAC	TGG	ATT	TCA	GGA	CAA	GAA	TAT	CTT	CAT	CAA	TCT	GCA	GAT	AAC	AAT	225		
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TCA	AAT	TAC	GGC	TTA	TCA	CCT	GAT	CGG	CAA	TTT	GTA	TAT	CTA	GAA	AGT	GAT	TAT	TCA	AAG	CTT	TGG	AGA	TAC	TCT	375		
TAC	ACA	GCA	ACA	TAT	TAC	ATC	TAT	GAC	CTT	AGC	AAT	GGA	GAA	TTT	GTA	AGA	GGA	AAT	GAG	CTT	CCT	CGT	CCA	ATT	450		
CAG	TAT	TTA	TGC	TGG	TCG	CCT	GTT	GGG	AGT	AAA	TTA	GCA	TAT	GTC	TAT	CAA	AAC	AAT	ATC	TAT	TTG	AAA	CAA	AGA	525		
CCA	GGA	GAT	CCA	CCT	TTT	CAA	ATA	ACA	TTT	AAT	GGA	AGA	GAA	AAT	AAA	ATA	TTT	AAT	GGA	ATC	CCA	GAC	TGG	GTT	600		
TAT	GAA	GAG	GAA	ATG	CTT	GCT	ACA	AAA	TAT	GCT	CTC	TGG	TGG	TCT	CCT	AAT	GGA	AAA	TTT	TTG	GCA	TAT	GCG	GAA	675		
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GAT	GAA	CGA	GTA	TGT	TTG	CAG	TGG	CTA	AAA	AGA	GTC	CAG	AAT	GTT	TCG	GTC	CTG	TCT	ATA	TGT	GAC	TTC	AGG	GAA	975		
GAC	TGG	CAG	ACA	TGG	GAT	TGT	CCA	AAG	ACC	CAG	GAG	CAT	ATA	GAA	GAA	AGC	AGA	ACT	GGA	TGG	GCT	GGT	GGA	TTC	1050		
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Q *																											5
AGA	ATT	AGC	ATT	GGA	AGC	TAT	CCT	CCA	AGC	AAG	AAG	TGT	GTT	ACT	TGC	CAT	CTA	AGG	AAA	GAA	AGG	TGC	CAA	TAT	1350		
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CTT	CAT	GAT	GGA	CGC	ACT	GAT	CAA	GAA	ATT	AAA	ATC	CTG	GAA	GAA	AAC	AAG	GAA	TTG	GAA	AAT	GCT	TTG	AAA	AAT	1500		
ATC	CAG	CTG	CCT	AAA	GAG	GAA	ATT	AAG	AAA	CTT	GAA	GTA	GAT	GAA	ATT	ACT	TTA	TGG	TAC	AAG	ATG	ATT	CTT	CCT	1575		
M I L P																											4
CCT	CAA	TTT	GAC	AGA	TCA	AAG	AAG	TAT	CCC	TTG	CTA	ATT	CAA	GTG	TAT	GGT	GGT	CCC	TGC	AGT	CAG	AGT	GTA	AGG	1650		
P	Q	F	D	R	S	K	K	Y	P	L	L	I	Q	V	Y	G	G	P	C	S	Q	S	V	R	29		
TCT	GTA	TTT	GCT	GTT	AAT	TGG	ATA	TCT	TAT	CTT	GCA	AGT	AAG	GAA	GGG	ATG	GTC	ATT	GCC	TTG	GTG	GAT	GGT	CGA	1725		
S	V	F	A	V	N	W	I	S	Y	L	A	S	K	E	G	M	V	I	A	L	V	D	G	R	54		
GGA	ACA	GCT	TTC	CAA	GGT	GAC	AAA	CTC	CTC	TAT	GCA	GTG	TAT	CGA	AAG	CTG	GGT	GTT	TAT	GAA	GTT	GAA	GAC	CAG	1800		
G	T	A	F	Q	G	D	K	L	L	Y	A	V	Y	R	K	L	G	V	Y	E	V	E	D	Q	79		
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I	T	A	V	R	K	F	I	E	M	G	F	I	D	E	K	R	I	A	I	W	G	W	S	Y	104		
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H	Y	K	N	S	T	V	M	A	R	A	E	Y	F	R	N	V	D	Y	L	L	I	H	G	T	179		
GCA	GAT	GAT	AAT	GTG	CAC	TTT	CAG	AAC	TCA	GCA	CAG	ATT	GCT	AAA	GCT	CTG	GTT	AAT	GCA	CAA	GTG	GAT	TTC	CAG	2175		
A	D	D	N	V	H	F	Q	N	S	A	Q	I	A	K	A	L	V	N	A	Q	V	D	F	Q	204		
GCA	ATG	TGG	TAC	TCT	GAC	CAG	AAC	CAC	GGC	TTA	TCC	GGC	CTG	TCC	ACG	AAC	CAC	TTA	TAC	ACC	CAC	ATG	ACC	CAC	2250		
A	M	W	Y	S	D	Q	N	H	G	L	S	G	L	S	T	N	H	L	Y	T	H	M	T	H	229		
TTC	CTA	AAG	CAG	TGT	TTC	TCT	TTG	TCA	GAC	TAA															2283		
F	L	K	Q	C	F	S	L	S	D																239		

Figure 3

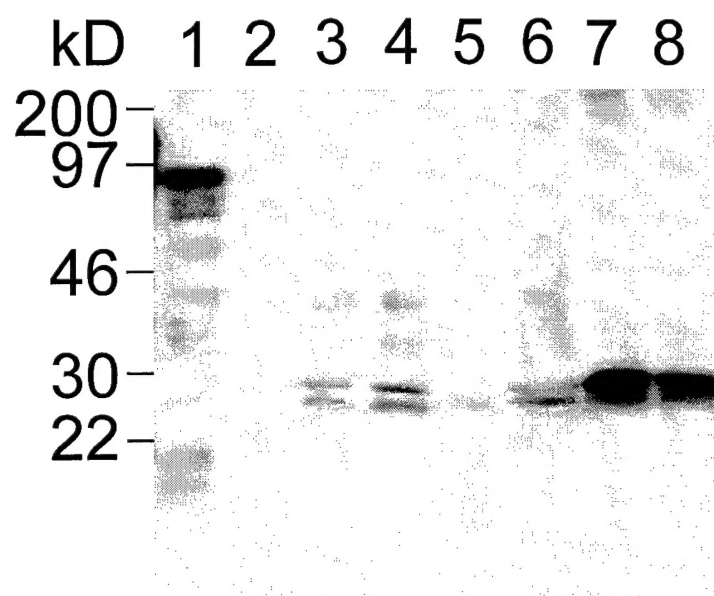


Figure 4

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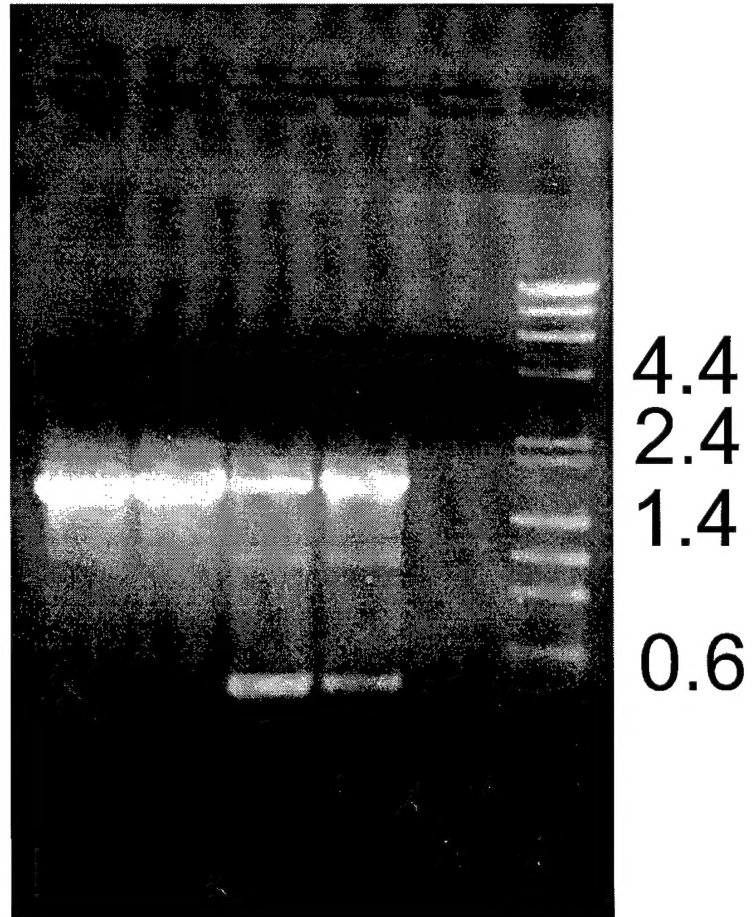


Figure 5

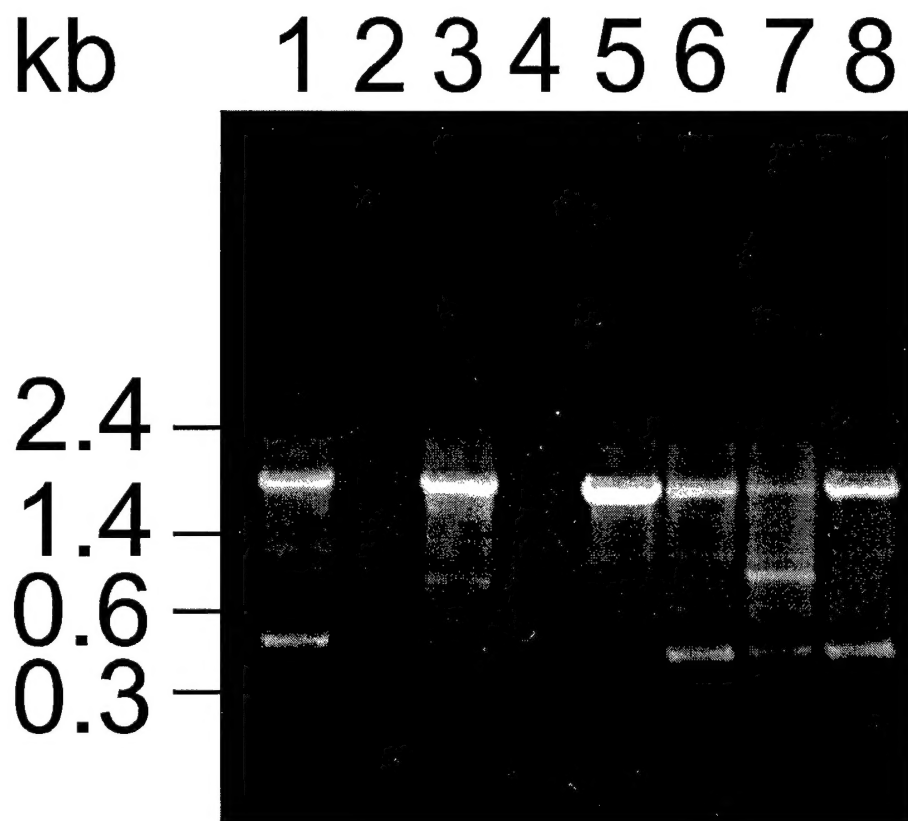


Figure 6

